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IN VITRO EXPERIMENT ON *LACTOBACILLUS CASEI* 01 COLONIZING THE DIGESTIVE SYSTEM IN THE PRESENCE OF PASTEURIZED LONGAN JUICE

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This study was to examine how encapsulated *Lactobacillus casei* 01 combined with pasteurized longan juice colonized a digestive system by using a simulator of the human intestinal microbial ecosystem. The results showed that encapsulated *L. casei* 01 and longan juice stimulated an increase of colon *lactobacilli*, *bifidobacteria*, lactic acid and various short-chain fatty acids for which acetate was substantially present in both colons followed by propionate and butyrate. On the contrary, the treatments triggered off the reduction of faecal coliforms, clostridia, and total anaerobes. To sum up, the denaturing-gradient-gel electrophoresis supported that treatment conditions stimulated diversities of bacteria communities occurring in both colons.

Keywords: encapsulated *Lactobacillus casei* 01, pasteurized longan juice, simulator of the human intestinal microbial ecosystem (SHIME), colon microbiota, metabolic products

To provide efficient functions, probiotics must survive through the upper gastrointestinal tract and be able to participate in the gut environment. Since these microorganisms are sensitive to several conditions, in particular acidic and bile fluids of the stomach and small intestine, encapsulating cells is necessary to protect them from various adverse environments. The most convincing procedure for encapsulation is coating in alginate beads by emulsion technique. This technique does not stress or harm the bacterial cells and can be easily performed in large volumes (DING & SHAH, 2008).

To examine the health-promoting effect of the probiotic, a simulator of the human intestinal microbial ecosystem (SHIME) was applied. A study of VAN DE WIELE and co-workers (2004) applied SHIME reactor to examine the impact of chicory inulin on colon microbiota and their metabolic activities. It was found that the growth of *lactobacilli* and *bifidobacteria* as well as the synthesis of butyrate and propionate significantly increased after the constant dosage of this prebiotic. Moreover, POSSEMIERS and co-workers (2010) harboured *Lactobacillus helveticus* CNCM I-1722 and *Bifidobacterium longum* CNCM I-3470 in the SHIME and observed the increase of *lactobacilli* and *bifidobacteria* counts in all colon compartments.

For decades, probiotics have been fortified in dairy products. However, the demand for non-dairy-based probiotic drink is increasing, because there is high volume of consumers who want to obtain not only health benefits from probiotics, but also beneficial nutrients from

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this soft-drink (YOON et al., 2004). Longan (*Dimocarpus longan* Lour.) juice is an alternative functional soft-drink containing bioactive components, such as ascorbic acid, gallic acid, ellagic acid, and phenolic compounds (CHAIKHAM & APICHARTSRANGKOON, 2012a, b). Therefore, this study aimed to investigate the health beneficial effects of longan juice plus *Lactobacillus casei* 01 on colon microflora and their metabolic products by applying SHIME reactor.

1. Materials and methods

1.1. Probiotic microencapsulation

Lactobacillus casei 01 was purchased from Chr. Hansen (Hørsholm, Denmark). The concentration of $\sim 10^{11}$ CFU ml⁻¹ was prepared and encapsulated in alginate beads following the method described by CHAIKHAM and co-workers (2012). The beads were prepared by emulsion technique, accordingly, the mixture of sodium alginate solution and diluted cells was dispersed in peanut oil plus Tween 80. The microbeads were formed by adding CaCl₂ into the mixture. After that, the collected beads were recoated with sodium alginate solution.

1.2. Preparation of pasteurized longan juice

Longan juice was prepared from Thai longan fruit (cv. Daw) and was subjected to pasteurization at 90 °C for 2 min. The juice (pH 6.5) comprised of 15% (w/v) sucrose, 0.2% (w/v) crude fibre, 250 mg/100 g minerals, 2.71 mg/100 ml ascorbic acid, 4.67 mg/100 ml gallic acid, 1.74 mg/100 ml ellagic acid, and 199.22 mg/100 ml total phenols (CHAIKHAM & APICHARTSRANGKOON, 2012a).

1.3. A simulator of the human intestinal microbial ecosystem

The SHIME reactor, a dynamic model of the human gastrointestinal tract, consists of four double-jacketed vessels simulating the stomach, small intestine, proximal colon, and distal colon, maintained at 37 °C under anaerobic conditions (VAN DE WIELE et al., 2007). It contains media used in the experiment, such as SHIME medium (a mixture of arabinogalactan, pectin, xylan, potato starch, glucose, yeast extract, special peptone water, mucin, and *L*-cysteine), pancreatic juice (a mixture of Oxgall, NaHCO₃, and pancreatin from porcine pancreas) and prepared-human-faecal fluid, for which the procedures of CHAIKHAM and co-workers (2012) were followed.

1.4. SHIME experiment

The SHIME was run continuously for 9 weeks with 3 treatment conditions and the wash-out periods. During the start-up period, 210 ml of acidified SHIME medium was fed into the first vessel (simulated stomach), then it was administered every on 8 h interval with a flow rate of 7 ml min⁻¹ until reaching 210 ml. The acidified SHIME medium was retained in the first vessel for 2 h, then transferred through the second vessel (simulated small intestine), which was previously filled with 90 ml pancreatic juice during the start-up period, then administered every on 8 h interval with a flow rate of 3 ml min⁻¹ until reaching 210 ml. This flow rate would enable a volume of 210 ml mixed fluid retaining to be retained in the second vessel for 4 h. After that the fluid from the vessel 2 was transferred to the proximal colon (vessel 3),

which was previously stabilized by SHIME medium plus faecal fluid. While the fluid from the small intestine was transferred through the proximal colon, some fluid in this colon was simultaneously transferred through the distal colon (vessel 4). Subsequently, some fluid from the distal colon was pumped out. The flow rate of the colon fluids (vessels 3 and 4) was adjusted to 1 ml min⁻¹. This system was operated continuously throughout the experiment.

The experimental set-up for various treatment conditions is shown in Table 1. During the treatment periods, the studied medium (210 ml SHIME medium or 105 ml SHIME medium+105 ml longan juice) with or without encapsulated *L. casei* 01 (~109 CFU ml⁻¹ in longan juice or SHIME medium) was fed once and the acidified SHIME medium was fed twice each day. Totally, the SHIME media were fed 3 times a day.

Table 1. Experimental set-up of SHIME reactor for long-term running

Experimental set-up	Feeding times (days)	Treatment compositions
Start-up	7	210 ml SHIME medium
Control	14	210 ml SHIME medium
Treatment 1	7	210 ml SHIME medium mixed with 10 g encapsulated <i>L. casei</i> 01
Treatment 2	7	105 ml SHIME medium mixed with 105 ml pasteurized longan juice containing 10 g encapsulated <i>L. casei</i> 01
Treatment 3	7	105 ml SHIME medium mixed with 105 ml pasteurized longan juice

1.5. Determination of metabolized products

Total lactic acid was quantified by the UV spectrophotometric method at a λ_{\max} 340 nm following CHAIKHAM and co-workers (2013).

Short-chain fatty acids (SCFA) were extracted as described by VAN ASSCHE (1978). The extract was then injected into a Di200 gas chromatograph (Shimadzu's-Hertogenbosch, The Netherlands) equipped with a flame ionization detector and a capillary free fatty acid-packed column. Nitrogen with a flow rate of 20 ml min⁻¹ was used as a carrier gas (CHAIKHAM et al., 2012).

1.6. Standard plate counting

Samples were plated on 5 types of selective media as follows; Lactobacillus Anaerobic MRS with Vancomycin and Bromocresol green agar for colon lactobacilli, Raffinose-Bifidobacterium agar for bifidobacteria, McConkey agar for total faecal coliforms, Tryptose Sulfite Cycloserine agar for total clostridia, and Brain Heart Infusion agar for total anaerobic bacteria. All plated media were subsequently incubated at 37 °C for colony counting.

1.7. Denaturing gradient gel electrophoresis (DGGE) analysis

The 16S rRNA genes of all bacteria were amplified applying primers P338F with a GC-clamp of 40 bp and P518r on total extracted DNA. Accordingly, DGGE was performed as described by MUYZER and co-workers (1993). Polymerase chain reaction (PCR) fragments were loaded onto 8% (w/v) polyacrylamide gels in 1 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4). After that, the polyacrylamide gels were made with denaturing gradient ranging from 45% to 60%, and then the electrophoresis was run for 16 h at 60 °C and 38 V.

1.8. Statistical data analysis

All data were the means of triplicate determinations with standard deviations (means±standard deviation). Analysis of variance (ANOVA) was carried out by using SPSS Version 15.0, and determination of significant differences among treatment means was done by Duncan's multiple range tests ($P \leq 0.05$).

2. Results and discussion

2.1. Formation of total lactic acids

Table 2 shows the formation of lactic acid in proximal and distal colons. Treatment 1 (encapsulated *L. casei* 01) induced significant increase ($P \leq 0.05$) of total lactic acids by 67.4% and 68.8% in proximal and distal colons, respectively, as comparison with the control. The effect of *L. casei* 01 was more pronounced with pasteurized longan juice as in treatment 2, leading to the increase of lactic acid by 157.5% and 139% in proximal and distal colons, respectively. These finding could be due to the large amount of carbon sources (15% sucrose) and some bioactive components in the longan juice. MACFARLANE and MACFARLANE (2003) stated that lactic acid was normally produced under excess of carbon, while BIALONSKA and co-workers (2010) found that gallic and ellagic acids in pomegranate by-product enhanced the growth of bifidobacteria and lactobacilli in the media inoculated with human faecal microflora. In addition, CHAIKHAM and co-workers (2012) noted that encapsulated *L. casei* 01 plus pressurized longan juice brought about the increase of lactic acid by 383%, 144%, and 43.6% in ascending, transverse, and descending colons, respectively. In this context, the pasteurized longan juice in treatment 3 also stimulated rising of lactic acid content by 128% and 110% in proximal and distal colons, respectively. Moreover, the amount of lactic acid gradually increased upon prolonging fermentation indicated in the distal colon.

Table 2. Formation of total lactic acids and quantities of colon lactobacilli in colon compartments

Experimental set-up	Proximal colon	Distal colon
Total lactic acids (g l^{-1} suspension)		
Control	135.56±9.23 ^d	154.66±4.33 ^d
Treatment 1	226.21±5.45 ^c	261.36±4.44 ^c
Treatment 2	348.99±9.19 ^a	370.32±2.42 ^a
Treatment 3	310.27±7.02 ^b	325.71±2.48 ^b
Colon lactobacilli (CFU ml^{-1} suspension)		
Control	6.89±0.54 ^d ×10 ⁴	3.81±0.16 ^c ×10 ⁴
Treatment 1	8.52±0.42 ^a ×10 ⁶	1.47±0.18 ^b ×10 ⁶
Treatment 2	3.82±0.21 ^b ×10 ⁶	4.67±0.23 ^a ×10 ⁶
Treatment 3	1.07±0.16 ^c ×10 ⁶	1.95±2.01 ^b ×10 ⁶

Means in the same column followed the same letters have no significant difference ($P > 0.05$). Each data point is the average of five replications

2.2. Colon lactobacilli

Table 2 shows that quantities of colon lactobacilli in proximal and distal colons significantly increased ($P \leq 0.05$) (from the control) by two log cycles after feeding treatment 1; however, the amount of colon lactobacilli dropped upon prolonging fermentation (in distal colon), presumably due to the accumulation of lactic acid retarding the growth rate. This phenomenon also applies to treatments 2 and 3. Nonetheless, treatment 2 gave rise to accumulating the highest number of colon lactobacilli at the final state of fermentation in the distal colon, which could be associated with the chemical composition of longan juice as discussed earlier.

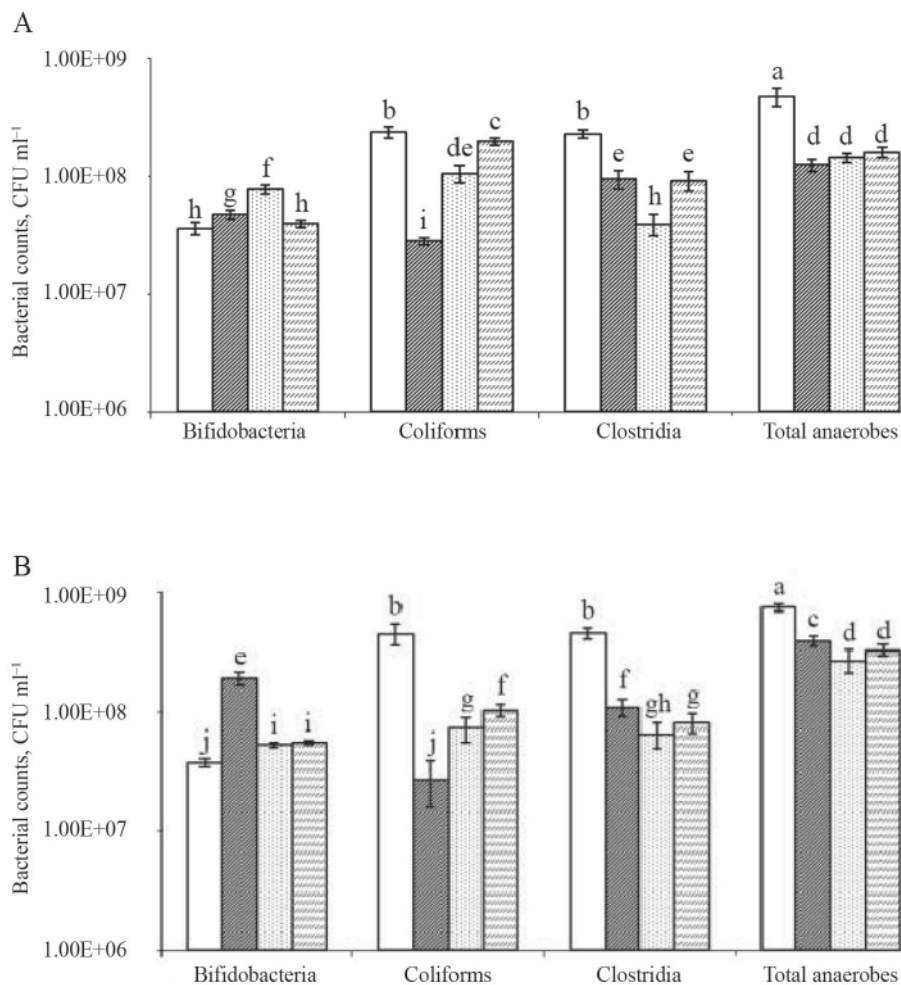


Fig. 1. Colon microbes determined in (A) proximal colon and (B) distal colon for various treatments: □: control; ▨: treatment 1; ▤: treatment 2; ▩: treatment 3. Means indicated with the same letters have no significant difference ($P > 0.05$). Each data point is the average of triplication

2.3. Other bacteria communities

Figure 1 shows the influence of various treatments on the survival of colon bacteria. All treatments in both colons triggered off a significant increase ($P \leq 0.05$) of beneficial bifidobacteria, but diminished some undesirable coliforms, clostridia, and total anaerobes in comparison with the controls. In addition, the impacts of treatment 2 were significantly prominent ($P \leq 0.05$) with clostridia. These results suggested that encapsulated *L. casei* 01 and pasteurized longan juice exerted the growth of bifidobacteria but suppressed the growth of harmful bacteria, which could be caused by the presence of antibacterial substances, such as fatty acids, organic acids, hydrogen peroxide, and/or bacteriocins. Usually, genera *Lactobacillus* and *Bifidobacterium* can produce bacteriocins to inhibit some pathogenic bacteria (MESSAOUDI et al., 2013).

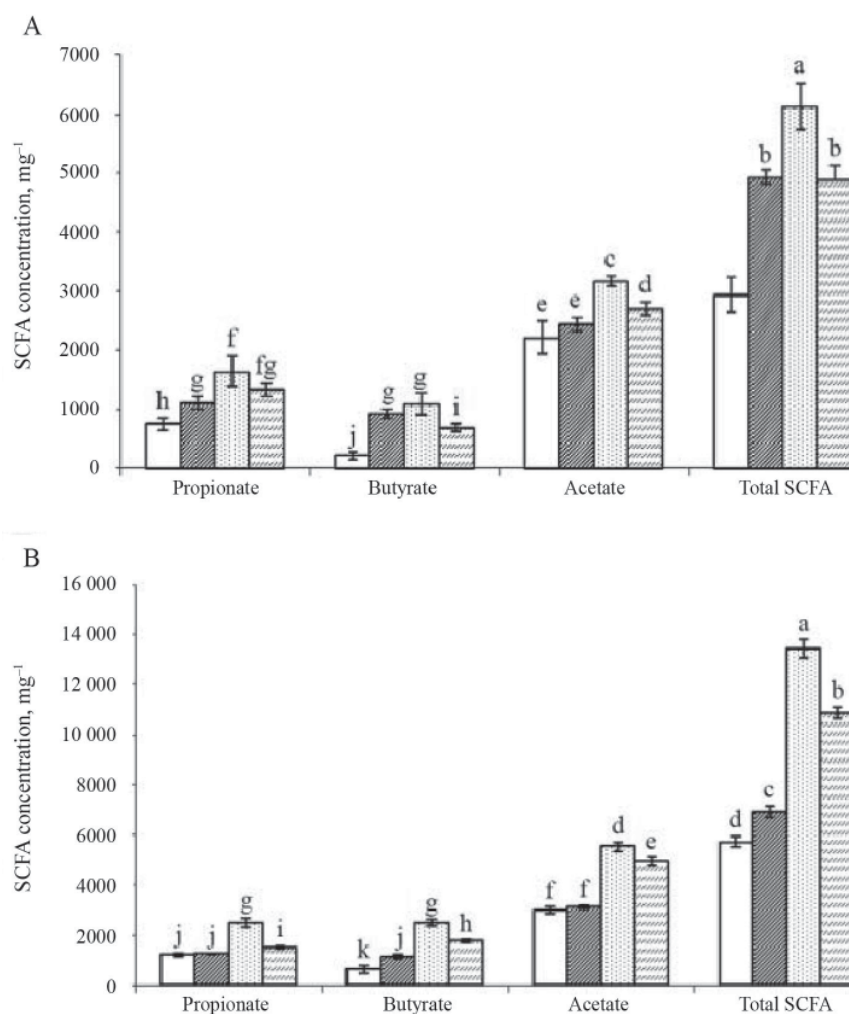


Fig. 2. Formation of short-chain fatty acids determined in (A) proximal colon and (B) distal colon for various treatments: □: control; ▨: treatment 1; ▩: treatment 2; and ▤: treatment 3. Means indicated with the same letters have no significant difference ($P > 0.05$). Each data point is the average of five replications

2.4. Formation of short-chain fatty acids

Figure 2 shows the formation of SCFA in both colon vessels. It was found that acetate, produced by lactobacilli and/or bifidobacteria (VAN DE WIELE et al., 2007), was the predominant SCFA in both colons and gradually increased with longer fermentation. Apart from acetate, propionate and butyrate were also detected in lower concentrations. LOUIS and co-workers (2007) reported that colon propionate and butyrate could be the converted products of acetate and lactate by bacteria such as *Anaerostipes caccae*, *Eubacterium halli*, *Megasphaera elsdenii*, and *Roseburia* spp. Moreover, encapsulated *L. casei* 01 and pressurized longan juice enhanced significantly the formation of total and other SCFA. Similar result was obtained by CUMMINGS and co-workers (1979), it showed that faecal SCFA formation was in the order of acetate > propionate > butyrate in a molar ratio of approximately 60:20:20, respectively. On the other hand, CHAIKHAM and co-workers (2012) fed *L. casei* 01 with pressurized longan juice into the SHIME reactor and found that the formation of propionate and butyrate was greater than that of acetate. In general, SCFA are the main energy source for colonocytes and contribute to several gut functions including carbohydrate and lipid metabolism. They also control colonic pH and maintain integrity of colonic mucosa, intestinal motility, or absorption (HIJOVA & CHMELAROVA, 2007).

2.5. Total microbial fingerprints

Figure 3 illustrates DGGE fingerprints of total colon bacteria with the influence of treatment conditions. Colon bacteria in the SHIME were classified into three groups as follows. The first group (G1) of bacteria was present in both colons in case of the control treatment. The second group (G2) of bacteria was also present in both colons in case of treatments 1, 2, and 3. The final group (G3) of bacteria was present in the proximal colon in case of treatment 3. These findings indicated that the bacterial groups in the treatments were different from the control, hence it confirmed that these treatments stimulated diversities of bacteria communities in both colons.

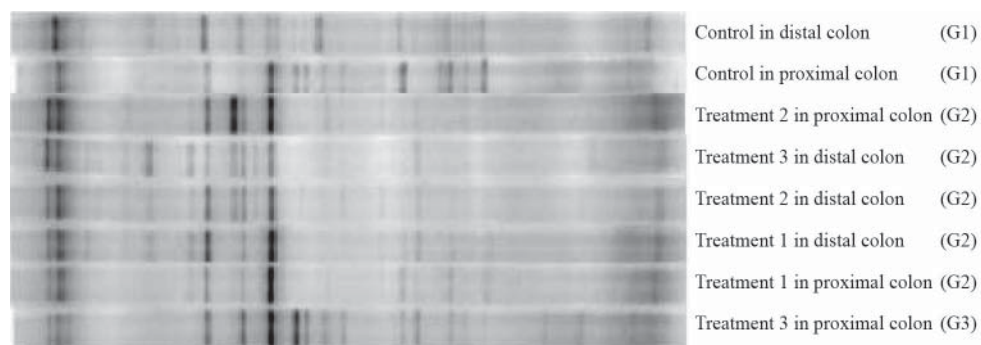


Fig. 3. DGGE fingerprint of total colon bacteria with the influence of encapsulated *L. casei* 01 plus pasteurized longan juice

3. Conclusions

The finding indicated that encapsulated *L. casei* 01 and pasteurized longan juice had significant impact on the increase of colon lactobacilli numbers, formation of lactic acid and SCFA, for which acetate was substantially present in both colons followed by propionate and butyrate. For other colon bacteria, encapsulated *L. casei* 01 and longan juice gave rise to the increase of bifidobacteria numbers, but diminished harmful bacteria such as coliforms, clostridia, and total anaerobes. The DGGE fingerprints also supported that treatment conditions enhanced diversities of bacteria communities occurring in both colons.

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